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PRINCIPAL INVESTIGATOR: Jasmine V Abella

CONTRACTING ORGANIZATION:
McGill University
Montreal
Quebec H3A 1A1
Canada

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Email: jasmine.abella@mcgill.ca

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Molecular Oncology Group, McGill University
687 Pine Ave west
Royal Victoria Hospital
Montreal H3A 1A1
Quebec
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The Hepatocyte Growth Factor (HGF) RTK, Met, regulates cell proliferation, differentiation, migration, invasion and survival. Met activation is tightly controlled through several levels of regulation to achieve an appropriate biological response. In addition to mutations that activate the Met receptor in human cancer, I have previously shown that the specific uncoupling of Met from ubiquitination results in its oncogenic activation through deregulate endocytosis. My recent work has uncovered a novel role for the Gab1 scaffold in regulating Met signaling, internalization and subsequent degradation. HGF stimulation induces membrane ruffling events required to initiate cell migration including the formation of lamellipodia and dorsal ruffles (DRs). I show that Gab1 localizes to and is required for DR formation and recruits the Met receptor to this plasma membrane compartment where receptors and various Gab1 binding signaling molecules become concentrated. Disruption of this signaling DR compartment severely alters Met dependent MAPK signaling profile and blocks cell migration. Paradoxically the Met receptor is also more efficiently internalized and subsequently degraded through DR and disruption of DR drastically delays Met degradation. This underscores the importance of understating RTK subcellular localization in addition to receptor stability and how this provides a further level of regulation on biological outcome. Interestingly, we also show that the endocytic protein, STAM2, can associate with Gab1 and is enriched in DRs with Gab1. This represents a novel function for both proteins in normal Met downregulation.

15. SUBJECT TERMS
Oncogenic receptor activation, modes of receptor internalization, Deregulated receptor degradation, Dorsal Ruffle formation. Cell migration. Signaling compartments

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Table of Contents

Page

INTRODUCTION

The Met receptor tyrosine kinase (RTK) and its ligand, Hepatocyte growth factor (HGF) are deregulated in human breast cancer, through over-expression, the acquisition of point mutations that elevate the Met receptor catalytic activity and co-expression of the ligand and receptor at the invasive tumour margins. We have shown that HGF stimulates the dissociation, motility and invasion of human breast cancer cell lines in culture. These are all processes that correlate with enhanced tumor growth and invasion. Deregulation of the Met receptor was generally thought to result in ligand-independent activation of the receptor, however, we have recently shown that escape from down-regulation constitutes another mechanism leading to RTK deregulation and that this mechanism may be common in human breast cancer (1).

Under normal conditions, Met downregulation involves receptor activation, ubiquitination, clathrin dependent endocytosis and degradation (2-4). I have demonstrated that receptor ubiquitination is not required for internalization, but is a signal necessary for efficient degradation (2). My next aim was to determine the requirements for Met receptor internalization and examine whether these events are deregulated in breast cancer. Studies on the EGFR (epidermal growth factor receptor) have clearly demonstrated that several internalization pathways are utilized by this receptor (5), However, it is not clear how each pathway regulates receptor signaling and degradation. These studies have not yet been carried out for the Met receptor, therefore it will be important to establish whether Met can internalize through clathrin-independent pathways, how they impact on Met signaling and stability and finally whether certain pathway(s) are selected for in Met overexpressing breast cancers.

BODY

(1) The Gab1 scaffolding protein is required for growth factor induced dorsal ruffles . Abella *et. al.* Manuscript in preparation.

HGF is a potent activator of cell migration and invasion (*6, 7*). This biological activity is principally due to the recruitment and phosphorylation of the scaffolding protein Gab1 by the Met receptor. Phosphorylation of Gab1 generates numerous phosphotyrosine docking sites for signalling molecules such as p85, Crk, PLCγ and Shp2 (*8*), which propagate growth factor signalling to induce changes in the actin cytoskeleton thereby promoting cell dispersal and migration. These processes are required to initiate an epithelial morphogenic program downstream from the Met receptor. Previous work by our group and others has demonstrated a requirement for Gab1 in cell dispersal, invasion and epithelial morphogenesis downstream from HGF in MDCK cells (*9-11*). Loss of Gab1 recruitment to the plasma membrane or to the Met receptor inhibits this program. Cell dispersal/scatter requires the reorganization of the actin cytoskeleton, which produces morphological changes in the plasma membrane such as the formation of peripheral ruffles and lamellopodia, to create a leading edge (*12*). We have previously shown that Gab1 localizes to lamellopodia and promotes their formation in MDCK cells (*13*). HGF stimulation of MDCK cells results in rapid formation of dorsal ruffles, also known as waves, which are actin rich tubular membrane protrusions. Although these dramatic membrane structures have been observed for decades, their function has largely remained elusive. Dorsal ruffles appear morphologically similar to macropinocytosis and, like the latter, are Rac dependent. However, unlike macropinocytosis, they form transiently and have been shown to require a distinct set of proteins to regulate their formation (*14*). Dorsal ruffles have been proposed to be a mechanism to enable a stationary cell to rapidly reorganize its actin cytoskeleton network in order to become motile (*12*). In addition, studies by McNiven's group have also demonstrated the localization of fluorescently tagged

EGF and PDGF receptors to these structures, suggesting that dorsal ruffles may also be a mechanism for receptor internalization(*15*).

In this chapter I demonstrate that Gab1 is essential for growth factor mediated dorsal ruffles. Depletion of Gab1 in MDCK cells severly impaired the ability of these cells to form dorsal ruffles in response to HGF. Similarly, stimulation of mouse embryonic fibroblast (MEF) cells null for Gab1 with EGF or PDGFbb, did not induce dorsal ruffle formation compared to parental lines stimulated with these ligands. Importantly, infection of the cells with GFP-Gab1 restored the ability of these cells to form dorsal ruffles in response to both EGF and PDGF. Furthermore, I show that overexpression of Gab1 can enhance HGF induced dorsal ruffles in MDCK cells and can promote their formation in HeLa cells, which do not readily form dorsal ruffles in response to HGF or EGF. Using both overexpression of Gab1 mutants in MDCK cells and via rescue experiments in the *Gab1*-/- null MEFs, I demonstrate that a Gab1 mutant uncoupled from the adaptor protein Crk is severely impaired in its ability to form dorsal ruffles in response to all three growth factors. However, neither loss of Crk nor overexpression of Crk could inhibit or enhance dorsal ruffle formation respectively, suggesting that recruitment of another protein other than Crk was important for Gab1 dependent dorsal ruffles. The Gab1ΔCrk mutant construct contains six tyrosine to phenylalanine substitutions (Y-F) and a survey of the motifs surrounding these tyrosines indicated that three also fit the consensus sequence for the SH2 domain of the adaptor protein Nck1 and 2. Importantly, Nck has been demonstrated to be required to promote Wave1 induced Arp2/3 actin polymerization, required for dorsal ruffle formation and cells null for Nck1 and 2 are unable to form dorsal ruffles in response to PDGFbb stimulation (*16*). Using a panel of Y-F mutants, I show that a single tyrosine residue is responsible for Nck 1 and 2 recruitment to Gab1 and that substitution of this tyrosine residue with phenylalanine is sufficient to uncouple Gab1 from Nck and importantly, inhibit the ability of Gab1 to induce growth factor mediated dorsal ruffles. Furthermore, Gab1ΔNck is also impaired in its ability to activate Rac downstream from HGF, which is essential for dorsal ruffle formation. This work demonstrates for the first time that Gab1 is a common requirement for dorsal ruffle formation downstream from multiple RTKs. I have also shown that mechanism of Gab1 mediated dorsal ruffles requires recruitment of Nck1 and 2, which are novel binding partners of Gab1 and disruption of this recruitment is sufficient to abrogate Gab1 mediated dorsal ruffles.

The biological function of dorsal ruffles is still poorly understood. However, several groups have proposed that dorsal ruffles may be required to induce cell dispersal and subsequent migration of cells in the context of a cell colony or tissue and secondly, dorsal ruffles may also represent a more efficient mechanism to downregulate activated RTKs. To address these hypotheses, I examined the affects of enhancing or inhibiting dorsal ruffle formation of cell migration and Met RTK half-life. Enhancing dorsal ruffle formation leads to faster trafficking and degradation of the Met receptor in both MDCK and HeLa cells. Similarly, induction of dorsal ruffles in HeLa cells through Pak1 over expression, a known to serine/threonine kinase required for dorsal ruffle formation, also results in more rapid Met degradation upon HGF stimulation. Loss of the Gab1 protein by siRNA severely reduces the number of dorsal ruffles formed in MDCK cells (by 60%). The stilbene drug SITS, which inhibits several ion exchangers, has also been shown to block dorsal ruffle formation in MDCK cells (*17*). We can show that SITS reduces the induction of dorsal ruffles in MDCK cells over expressing Gab1 by almost 8 fold. Ablation of these structures using SITS also delays Gab1 phosphorylation, Met degradation, severely alters the MAPK pathway activation profile and inhibits cell scatter. We can show that localization of Gab1 to dorsal ruffles is also accompanied by localization of signalling proteins such as Erk1/2 and PLCγ. Our data provides a novel role for Gab1 in promoting dorsal ruffle formation downstream from multiple RTKs, and suggests that dorsal ruffles act as a specialized Gab1 signalling compartment and novel internalization route required for biological activities such as cell scatter.

RTK internalization through dorsal ruffles would represent an efficient and fast mechanism to rapidly degrade receptors. Paradoxically, our results show that enhanced degradation does not correlate with an inhibition of cell signaling, but rather an alteration of signaling profiles thereby changing biological outcomes. This demonstrates that subceullar localization plays a key role in determining signaling outcome and further underscores the importance of understanding receptor subcellular localization in addition to receptor stability. We have demonstrated that dorsal ruffles represent a novel signaling compartment required for HGF induced biological functions such as cell migration. Importantly, tumor derived cell lines from mice overexpressing the Met receptor created by our group exhibit an enhanced capacity to form dorsal ruffles and have elevated Nck protein levels. The mechanism by which dorsal ruffles can promote cell migration and invasion still remains to be determined.

Part of this data was originally submitted to the EMBO Journal in October 2008 (see appendix 1). I have now addressed the reviewers' comments and am preparing this manuscript for two new submissions to EMBO Journal and Traffic in April 2009.

(2) A novel role for the endocytic adaptor protein STAM in dorsal ruffle formation. Abella *et.al.* manuscript in preparation.

Epithelial morphogenesis requires cell dispersal and migration and these processes are initiated through changes in the actin cytoskeleton which alter the structure of the plasma membrane. Plasma membrane ruffling has been demonstrated to require new membrane from endosomal vesicles (*20*). However, the events and players which regulate how and where endosomes fuse with the plasma membrane are still poorly understood. Rab5, a small GTP binding protein which localizes to early endosomes, has previously been reported to be required for dorsal ruffle formation (*21, 22*). Recently, a genetic screen by Affolter's group identified another endocytic adaptor molecule, STAM (Signal Transducing Adaptor Molecule), as a key regulator in tracheal cell migration during Drosophila air sac morphogenesis. STAM has previously been shown to function with Hrs on Rab5 endosomes, to recognize ubiquitinated RTKs and facilitate their lysosomal degradation. I have shown that STAM can associate with Gab1 and following my previous work on Gab1 function is dorsal ruffles, the aim of this last chapter will be to determine the functional relevance of this interaction with respect to membrane ruffling and cell migration.

STAM contains an atypical SH3 domain which recognizes PX(V/I)(D/N)RXXKP motifs. Our lab has previously demonstrated that Gab1 contains an atypical proline rich motif which is recognized and bound by an SH3 domain of Grb2 (*23*). I have shown that this atypical proline rich motif in Gab1 is a substrate for the SH3 domain of STAM both through over expression in 293 cells and with the endogenous proteins in HeLa cells. STAM has previously only been reported to localize to endosomes, however I have shown that a pool of STAM localizes with Gab1 at the plasma membrane and is also recruited to dorsal ruffles in MDCK cells upon HGF stimulation. Interestingly, a mutant construct of STAM lacking the SH3 domain (STAMΔSH3) and therefore unable to associate with Gab1, is still able to localize to the plasma membrane but to a lesser extent, suggesting a Gab1 independent mechanism of targeting STAM to the plasma membrane. In addition, live cell imaging of MDCK GFP Gab1 stable cells co-transfected with mCherry STAMΔSH3, showed reduced membrane ruffling compared to cells expressing only GFP Gab1. Importantly, depletion of STAM 1 and 2 in MDCK cells by siRNA severely abrogated HGF induced dorsal ruffle formation. Interestingly, this correlated with an inhibition in cell migration using boyden chamber assays under conditions of STAM1/2 knockdown.

To address whether STAM functions in RTK internalization from dorsal ruffles, I will perform live cell imaging analysis to determine if Met localizes with STAM on dorsal ruffles and assay if STAM proteins are required for Met recruitment into and internalization from dorsal ruffles by live cell microscopy. STAM is differentially phosphorylated downstream from a wide range of RTKs and G-Protein coupled receptors and therefore may represent a common mechanism to induce receptor internalization through dorsal ruffles.

This manuscript is under preparation for submission to Traffic in June 2009.

(3) Determine the consequence of altered Met subcellular localization in human breast cancer cell lines.

I have not been able to start this project due to time constraints but was able to establish all conditions for confocal microscopy analysis of Met subcellular localization and will be handing this project over to a new postdoctoral fellow.

KEY RESEARCH ACCOMPLISHMENTS

The Gab1 scaffolding protein localizes to and is required for dorsal ruffles formation downstream from multiple receptor tyrosine kinases.

Gab1 mediated dorsal ruffles requires recruitment of Nck1 and 2, which a novel Gab1 interacting proteins.

Dorsal Ruffles represent a novel signaling compartment, which if disrupted, alters the signaling profile of the MAPK pathway and blocks HGF induced cell migration.

Gab1 has a novel role in Met down regulation by enhancing Met degradation.

Gab1 mediated Met degradation occurs through a novel route of Met internalization into the cell, through dorsal ruffles.

STAM is a novel Gab1 interacting protein, which localizes to dorsal ruffles with Gab1, is required for dorsal ruffle formation and HGF induced cell migration. This work signifies a novel, non-endocytic function for the adaptor protein STAM 1 and 2 .

REPORTABLE OUTCOMES

Manuscripts

- **ABELLA JV**, Peschard P, Naujokas MA, Lin T, Saucier C, Urbe S and Park M,. Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol. Cell. Biol., 2005, Nov:25(21) 9632-45.

- Sangwan, V, Paliouras GN*, **ABELLA JV***, Zuo D, Dube N, Tremblay ML and Park M, (2008). *Regulation of the Met receptor tyrosine kinase by the protein tyrosine phosphatases PTP1B and TCPTP*. J Biol Chem., Dec 5; 283 (49): 34374-83. (* These authors contributed equally).

- **ABELLA JV** and Park M. Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases. Am J Physiol Endocrinol Metab. 2009 Feb 24. [Epub ahead of print].

-Lai A, **ABELLA JV** and ParkM. Cross Talk in Met Receptor Oncogenesis. Invited review for Trends in Cell Biology, submitted in March 2009.

Invited Oral Presentations

-ABELLA JV, Parachoniak C., Zuo D., Park M., *A novel role for the Gab1 scaffolding protein in mediating internalization of the Met receptor tyrosine kinase through circular ruffles.* Cold Spring Harbor Meeting on Phosphorylation, Signaling and Disease. May 2007

Abstracts (since award start date)

- ABELLA JV, Frigault MM, Parachoniak C, Sangwan and Park M. (July 2008) *The Gab1 scaffold coordinates growth factor induced dorsal ruffles and receptor degradation*. Breast Cancer Research Program (BCRP) Era of Hope 2008 Meeting. Baltimore, USA,.

-ABELLA JV*., Parachoniak C., Zuo D., Park M., (May 2007). *A novel role for the Gab1 scaffolding protein in mediating internalization of the Met receptor tyrosine kinase through circular ruffles.* Poster to be presented at the Cold Spring Harbor Meeting on Phosphorylation, Signaling and Disease.

-ABELLA JV*., Frigault M., Park M., (July 2006). *The role of the Gab1 scaffold protein in downregulation of the Met receptor tyrosine kinase*. Poster at Gordon Research Conference on Lysosomes and Endocytosis.

Awards

Nominated by the Department of Biochemistry at McGill University for the Principal's Dissertation Award. November 2007.

First Prize for Poster Presentation at Workshop in Endocytic Systems, Villars-sur-Ollons, Switzerland. September 2007,

CONCLUSION

How signal output is regulated by the mode of receptor internalization and subcellular localization, is still an area that we know little about. My work has focused on trying to understand how the Met receptor internalizes and traffics within the cell under normal conditions and how deregulation of the receptor can be caused by aberrant subcellular localization. I have previously demonstrated that deregulation of the Met receptor, through loss of Cbl mediated ubiquitination, results in a receptor that exhibits altered trafficking, escapes degradation and induces sustained signaling of the MAPK pathway, leading to cell transformation (2). The data presented in this report demonstrates a novel mode of entry into the cell for the Met receptor, mediated by the Gab1 scaffolding protein, through circular dorsal ruffles or CDRs. Further characterization of this pathway revealed that two endocytic adaptor proteins, Hrs and STAM, only known to function at the level of the sorting endosome, associate with Gab1 and are present on CDRs. This may represent a novel complex involved in targeting Met and perhaps other RTKs for internalization through CDRs. This pathway leads to enhanced degradation of the Met receptor compared to that downstream from clathrin mediated internalization. Therefore deregulation of this pathway could lead to enhanced stability and signaling of Met and other RTKs. Future work will determine whether breast cancer cell lines which express high levels of Met have selected against this faster mode of RTK degradation. I will also determine whether the aberrant subcellular localization of

Met that has been observed in these cell lines is due to mutations within the Met receptor, or a defect in the endocytic machinery, which would impact on all receptors utilizing this pathway.

Our understanding of the regulatory events involved in mediating the trafficking and degradation of Met and other RTKs, and how these are altered in human breast cancer will impact on our understanding of the molecular mechanisms of breast cancer.

"SO WHAT"

Treatment strategies that are currently used on breast cancer patients with EGFR family positive tumors, involve the use of monoclonal antibodies (mAbs) such as Herceptin against HER2 and Cetuximab against EGFR. These mAbs target the receptor at the plasma membrane and function by inducing ligand-independent receptor internalization and degradation through the endocytic pathway, terminating sustained receptor signaling (*28*). Our lab has observed aberrant localization of the Met receptor in several breast cancer cell lines, where the majority of the receptor is present within the cell and not at the plasma membrane. Hence, cases where RTKs become misslocalized to a subcellular location, will be refractory to such treatments targeted to the extracellular domain at the cell surface. In addition, the drug gefitinib (Iressa), a specific small molecule inhibitor of EGFR tyrosine kinase, was found to be effective in suppressing the survival and proliferation in some but not all non-small cell lung cancer (NSCLC) cell lines, by enhancing the rate of EGFR trafficking and degradation (*29*). Work by Ono's group demonstrated that the cell line insensitive to gefitinib treatment harbored a defect in the endocytic machinery, preventing efficient degradation (*29*). The concentration of ligand available to receptors may also influence the endocytic route taken and therefore the signaling outcome (5). The hypothesis that deregulation of RTKs through altered endocytosis, is clearly being validated now with work from several groups reporting altered EGF and Met receptor trafficking in cancer cells (*30, 31*). Thus treatment strategies to induce degradation through the normal endocytic pathway will be ineffective in situations where the trafficking pathway itself is deregulated. It is therefore crucial to understand how receptors traffic and to identify the subcellular locations within the cells where aberrant RTK signaling is occurring. This will allow for the design of potentially more effective drug treatments that target the receptor at the appropriate cellular location.

Targeted therapies against the Met receptor are in development and studies suggest that such a drug would hold much promise in cancer treatment. The Met receptor is implicated in all stages of cancer progression, including proliferation, invasion and metastasis. It is also expressed in endothelial cells, and would allow therapies to not only target cancer epithelium, but the tumor microenvironment as well, which is believed to be important in tumorigenesis. Studies on Met receptor trafficking, subcellular localization and deregulated signaling will thus contribute to the design of effective Met-targeted drugs.

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The Gab1 scaffold is required for RTK signal polarisation to dorsal ruffles

Jasmine V. Abella [1,2], Melanie M. Frigault [1,2], Christine A. Parachoniak [1,2], Veena Sangwan [2,] and Morag Park [1,2,3,]*.

Department of Biochemistry[1], Medicine[3], and Oncology[4], McGill University, Montréal, Québec, Canada; Molecular Oncology Group[2], McGill University, Montréal, Québec H3A 1A1, Canada [2].

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Key words: Gab1, RTK, Dorsal Ruffles, Endocytosis, Receptor Down-regulation.

*Corresponding Author. Mailing address: Molecular Oncology Group H5.21, McGill University Health Centre, 687 Pine Ave. West, Montréal, Québec, Canada H3A 1A1. Phone: (514) 398 5074, Fax 514-398-6769. E-mail: morag.park@mcgill.ca.
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Corresponding Author	Dr. Morag Park (McGill University)
Contributing Authors	Jasmine Abella , Melanie Frigault , Christine Parachoniak , Veena Sangwan
Abstract	How signalling and biological response to the hepatocyte growth factor (HGF) receptor tyrosine kinase (RTK), Met, are coupled to Met trafficking is largely unknown. The Gab1 scaffold protein modulates Met signals involved in cell dispersal and morphogenesis. We show that Gab1 is indispensable for a form of RTK-induced actin remodelling, called dorsal ruffles, in response to HGF, epidermal and platelet derived growth factors. Localisation of Gab1 and activated Met to dorsal ruffles is accompanied by signalling proteins recruited to Gab1. Structure-function demonstrates a requirement for Gab1-Crk complexes for dorsal ruffle formation. Gab1 induced dorsal ruffles promote a polarised signalling microenvironment from which Met is bulk internalised and degraded. Ablation of dorsal ruffles delays Met degradation but diminishes biological responses. We demonstrate an essential role for Gab1 in dorsal ruffle formation by multiple RTKs and provide direct evidence that dorsal ruffles act as a biologically relevant signalling microenvironment and mechanism for receptor down-regulation.
Keywords	Gab1, RTK, Dorsal Ruffles, Endocytosis, Receptor doen-regulation

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Summary

How signalling and biological response to the hepatocyte growth factor (HGF) receptor tyrosine kinase (RTK), Met, are coupled to Met trafficking is largely unknown. The Gab1 scaffold protein modulates Met signals involved in cell dispersal and morphogenesis. We show that Gab1 is indispensable for a form of RTK-induced actin remodelling, called dorsal ruffles, in response to HGF, epidermal and platelet derived growth factors. Localisation of Gab1 and activated Met to dorsal ruffles is accompanied by signalling proteins recruited to Gab1. Structure-function demonstrates a requirement for Gab1-Crk complexes for dorsal ruffle formation. Gab1 induced dorsal ruffles promote a polarised signalling microenvironment from which Met is bulk internalised and degraded. Ablation of dorsal ruffles delays Met degradation but diminishes biological responses. We demonstrate an essential role for Gab1 in dorsal ruffle formation by multiple RTKs and provide direct evidence that dorsal ruffles act as a biologically relevant signalling microenvironment and mechanism for receptor down-regulation.

Introduction

Hepatocyte growth factor and its receptor tyrosine kinase, Met, are potent activators of epithelial cell dispersal, morphogenesis and invasive growth (Birchmeier et al, 2003). This biological activity is principally due to the recruitment and phosphorylation of the scaffold protein Gab1 (Grb2-associated binder-1) by the Met receptor (Lock et al, 2002; Maroun et al, 1999b; Sachs et al, 2000; Weidner et al, 1996). Phosphorylation of Gab1 by Met, generates numerous phosphotyrosine docking sites for signalling molecules that includes the p85 subunit of PI3' kinase, the Crk adaptor protein and the tyrosine phosphatase, Shp2 (Gual et al, 2000; Lamorte et al, 2002b; Maroun et al, 1999b). Dispersal of colonies of epithelial cells in response to HGF requires the breakdown of cell-cell junctions and reorganization of the actin cytoskeleton. These are associated with morphological changes in the plasma membrane including formation of ruffles and lamellipodia (Royal et al, 2000; Royal & Park, 1995). Unlike lamellipodia, ruffles are sheet-like membrane protrusions which lack adherence to the substratum. Two distinctive forms of membrane ruffling events have been reported, peripheral ruffles and circular dorsal ruffles or waves (Abercrombie et al, 1970).

Stimulation of colonies of epithelial Madin-Darby Canine Kidney (MDCK) cells with HGF, promotes rapid formation of dorsal ruffles, which are F-actin rich circular membrane protrusions (Dowrick et al, 1993). Dorsal and peripheral ruffles form in response to stimulation by growth factors such as EGF, PDGFβ and HGF (Buccione et al, 2004; Dowrick et al, 1993). However, dorsal ruffles are distinct from peripheral ruffles in their temporal regulation and localisation (Buccione et al, 2004) and peripheral ruffles do not appear to convert into dorsal ruffles (Araki et al, 2000). While peripheral ruffles can occur continuously upon growth factor stimulation, are required for macropinocytosis and cell migration (Ridley et al, 1992; Suetsugu et al, 2003), dorsal ruffles form only transiently within the first 20 minutes of stimulation (Buccione et al, 2004) and their specific function is poorly understood.

In response to growth factor stimulation, dorsal and peripheral ruffle formation is dependent on Rac activation (Krueger et al, 2003; Lanzetti et al, 2004; Palamidessi et al, 2008; Ridley et al, 1992; Suetsugu et al, 2003). Each utilize common signals involving the Arp2/3 complex, cortactin and palladin, which are required for actin polymerization, as well the GTPases dynamin (Goicoechea et al, 2006; Krueger et al, 2003; Liu et al, 2007; McNiven et al, 2000). Specific activation of the Arp2/3 complex in the context of dorsal ruffles can be mediated by Rac effectors, N-WASP and WAVE (Krueger et al, 2003; Suetsugu et al, 2003; Westphal et al, 2000), where WAVE-1 is required for dorsal, but not peripheral ruffle formation (Suetsugu et al, 2003). Over-expression of activated Rac alone is not sufficient to induce dorsal ruffle formation, although it can promote the formation of peripheral ruffles, indicating that proteins which regulate the localisation of activated Rac such as the small GTPase Rab5 are critical (Lanzetti et al, 2004; Palamidessi et al, 2008). The serine/threonine kinase Pak1, an effector of Rac has also been demonstrated to induce dorsal ruffles downstream from the PDGF receptor (Dharmawardhane et al, 2000). Similarly, inhibitors of PI3’ kinase , Src and PLCγ disrupt dorsal ruffle formation in response to growth factors (Dharmawardhane et al, 1997; Mettlen et al, 2006; Veracini et al, 2006) However, how RTKs coordinate these processes is poorly understood.

Dorsal and peripheral ruffles in response to growth factors, are thought to function as sites for macropinocytosis, mediatiating nutrient uptake as well as a mechanism to recycle plasma membrane and membrane components (Dharmawardhane et al, 2000; Dowrick et al, 1993; Jones, 2007; Mettlen et al, 2006). However, fibroblasts deficient for WAVE-1 protein, form peripheral, but not dorsal ruffles in response to PDGFβ and still support macropinocytosis,

indicating that dorsal ruffles are not essential (Suetsugu et al, 2003). In response to EGF, the EGFR localises to dorsal ruffles and the subsequent internalisation of the EGFR from these structures has lead to the proposal that dorsal ruffles may also provide a mode for bulk internalisation of the EGFR (Orth et al, 2006). Internalisation of RTKs into the endosomal trafficking compartment is required for their subsequent degradation in the lysosome and provides one of the major mechanisms to regulate RTK signalling, stability and hence biological activity. In general, this is mediated through the entry of RTKs through clathrin coated pits, although other mechanisms for RTK entry have been proposed (Mayor & Pagano, 2007). However, the possibility that dorsal ruffles may provide a specialised membrane microdomain in which RTK signalling and activity are regulated remains poorly understood. Here we have addressed the molecular requirements for RTK induced dorsal ruffles and have analyzed their impact on receptor signalling and stability. We demonstrate that the Gab1 scaffold protein is a common requirement for RTK mediated dorsal ruffles. We show that Gab1 associated signalling molecules localise to these structures with activated Met receptors to form a biologically relevant signalling microenvironment which in turn modulates receptor down-regulation.

Results

HGF induced dorsal ruffles form in cell colonies and contain Met signalling complexes.

Regulators of dorsal ruffle formation downstream from growth factor receptor tyrosine kinases have been characterized in fibroblasts or tumor cells that grow as single cells (Krueger et al, 2003; Lanzetti et al, 2004; Orth et al, 2006; Suetsugu et al, 2003). To gain insight into the biological consequence of dorsal ruffles, we examined the regulation of dorsal ruffle formation and their requirement for signalling and biological response downstream from the Met receptor in colonies of well-polarized epithelial sheets which reflect a more physiological environment. Stimulation of colonies of MDCK cells with physiological concentrations of HGF (Funakoshi & Nakamura, 2003), induces the formation of dorsal ruffles in cells throughout the colony; visualized as concentric actin rich rings on the apical cell surface (Figures 1A, solid arrows and S1A) (Dowrick et al, 1993). Indirect immuno-fluorescence reveals that Met and the Met substrate, Gab1, are present on HGF induced dorsal ruffles (Figure 1B-C). Live cell imaging of MDCK cells over-expressing GFP tagged Gab1 (MDCK GFP-Gab1), demonstrates that in response to HGF, Gab1 is rapidly recruited to dorsal ruffles, where each dorsal ruffle persists for approximately 8 minutes. These structures can be initiated as early as 1 minute post stimulation, and can continue to form up to 20 minutes after HGF treatment, in cells both within and at the edge of a colony (Movie 1). Notably, each cell within the colony can produce more than one dorsal ruffle at any time (Figure 1A, solid arrows and movie 1), and these can occur independently of the ability for cells to form lamellipodia (Figure 1A, dashed arrow), which occur only at the edge of a colony.

The prolonged localisation of both Met and Gab1 in dorsal ruffles (up to 8 minutes), suggests that these structures are an active signalling microenvironment in which Gab1 can couple activated Met receptors with downstream signalling molecules. Using phospho-specific antibodies that reflect activation of the Met receptor (pY1234/35), we show the presence of activated Met and Gab1 in dorsal ruffles (Figure 1C). Signalling molecules recruited to Gab1 following Met activation, including phospho-Erk1/2, Crk, Shp2 and p85, also localise to dorsal ruffles in response to HGF stimulation consistent with this structure reflecting an active signalling compartment (Figures 1C-D and S1B-D).

Gab1 is required for, and enhances HGF dependent dorsal ruffle formation.

To determine if Gab1 dependent functions contribute to the formation of dorsal ruffles we examined the consequence of Gab1 knock down using siRNA, as well Gab1 over-expression. Using three independent siRNA duplexes to knock down Gab1, we show a statistically significant decrease (*) in the formation of dorsal ruffles in MDCK cells in response to HGF. Up to 4 fold fewer dorsal ruffles were observed with a knock down of 62% in Gab1 protein levels (Figure 2A and S1E-F). In contrast, over-expression of Gab1 in MDCK cells increases the incidence of dorsal ruffle formation in response to HGF by more than two fold, where approximately 60% of MDCK GFP-Gab1 cells form dorsal ruffles when compared to approximately 25% of wild-type MDCK cells at any one time (Figures 2B-D). Notably, in HeLa cells which do not readily form circular dorsal ruffles in response to low levels of HGF (Figure 2E, cell denoted by *), transient over-expression of Gab1 promotes the formation of dorsal ruffles in response to HGF (Figure 2E and Movie 2). Together, these results demonstrate that Gab1 dependent signals mediate dorsal ruffle formation downstream from the Met receptor.

Gab1 is essential for dorsal ruffles downstream from EGF and PDGF receptors.

To examine whether Gab1 is required for dorsal ruffle formation downstream from other RTKs which also phosphorylate Gab1 (Gu & Neel, 2003), we determined the ability of mouse embryonic fibroblast (MEF) cells, isolated from *Gab1* knock out or wild-type embryos, to form dorsal ruffles (Holgado-Madruga & Wong, 2003) (Figure S2A). Stimulation of wild-type (wt) MEF cells with either EGF or PDGFβ promoted dorsal ruffle formation as visualized by staining for actin and cortactin, established markers for dorsal ruffles (McNiven et al, 2000) (Figure 3A). In response to PDGFβ, approximately 40% of wt MEF cells form dorsal ruffles whereas only 15% (7C) and 10% (2B) of cells null for *Gab1* form dorsal ruffles (Figures 3A-B). Similarly, EGF induced dorsal ruffle formation was drastically impaired in the absence of Gab1, where approximately 30% of wt MEF cells form dorsal ruffles in response to EGF whereas only 8% (7C) and 7% (2B) of *Gab1*-null MEF cells form EGF dependent dorsal ruffles (Figures 3A-B). Importantly, stable re-expression of GFP-Gab1 in *Gab1*-null MEF cells rescued the dorsal ruffle response to both EGF and PDGFβ (Figure 3C). In addition, expression of Gab1 in HeLa cells promoted EGF induced dorsal ruffles (Figure S2B). Together, this demonstrates that Gab1 is required for dorsal ruffle formation downstream from the HGF, EGF and PDGF receptors.

A Gab1-Crk complex is required for Gab1-dependent dorsal ruffle formation.

Having established that Gab1 is required for dorsal ruffle formation downstream from multiple RTKs, we sought to determine the molecular requirements for this Gab1 function. To this end, we employed a structure-function approach in HeLa cells, using Gab1 constructs impaired in their recruitment of different signalling molecules (Lamorte et al, 2002b; Maroun et al, 1999b; Maroun et al, 2000). We show that Gab1 mutants unable to recruit the adaptor molecule Crk, or that lack the pleckstrin homology (PH) domain, fail to induce dorsal ruffle formation in response to HGF stimulation in the presence of serum, whereas mutants lacking recruitment of the p85 subunit of PI3K or Shp2 promote dorsal ruffles to a similar extent as wt Gab1 (Figures 4A and S2C). The Gab1ΔPH mutant is readily phosphorylated in response to HGF, but fails to localise to PIP_3 rich membrane micro-domains in response to HGF (Maroun et al, 1999a), indicating both a requirement for Gab1 subcellular localisation as well as recruitment of Crk for its ability to promote dorsal ruffles.

Although multiple Gab1 binding proteins are recruited to dorsal ruffles, generating a unique signalling microenvironment, only the Gab1-Crk interaction is required for the induction of dorsal ruffles. MDCK cells stably over-expressing a Gab1ΔCrk mutant, were impaired by at least 90% in their ability to form dorsal ruffles when compared to cells expressing either Gab1, Gab1Δp85 or Gab1ΔShp2 (Figure 4C), suggesting that the Gab1ΔCrk mutant, which is efficiently recruited and phosphorylated by the Met receptor, interferes with recruitment of endogenous Gab1 to Met (Lamorte et al, 2002b). Over-expression of CrkII alone however, was not sufficient to enhance dorsal ruffle formation in MDCK cells, demonstrating a specific requirement for Gab1-Crk interaction as well as Gab1-PIP_3 membrane associations, for Met induced dorsal ruffle formation (Figures 4D and S2D).

Increased dorsal ruffles enhance HGF induced Met receptor degradation.

One of the predominant mechanisms of RTK down-regulation is mediated through ligand induced receptor internalisation into the endocytic pathway, leading to lysosomal degradation of the receptor (Wiley & Burke, 2001). Concentration of RTKs in dorsal ruffles has been proposed to allow bulk internalisation of activated receptors following ligand stimulation (Orth et al, 2006),

however this has not been addressed biochemically. To determine if HGF-dependent Met down-regulation is altered by dorsal ruffle formation, we measured the steady state levels of Met post HGF stimulation in MDCK cells, with and without Gab1 over-expression. Notably, in MDCK cells over-expressing Gab1, which form an increased number of dorsal ruffles, degradation of the Met RTK is enhanced significantly (Figure 5A). The half-life of Met was 24.3±2.1 minutes in cells that over-express Gab1, when compared to 71.5±5.8 minutes in MDCK control cells. Enhanced Met degradation requires HGF stimulation, as increasing levels of Gab1 over-expression is not sufficient to induce Met degradation alone (Figure S3A). This implies that Met is efficiently endocytosed from the dorsal ruffle microenvironment to a degradative compartment (Figure 5B). Consistent with this, Met positive puncta are observed on the dorsal ruffle membrane at early time points and at the base of the ruffle at later time points (Figure 1B). In addition, rapid trafficking of Met positive endosomes to a peri-nuclear compartment was observed in response to HGF in Gab1 over-expressing MDCK cells when compared to control cells (Figure 5C). Efficient degradation of the Met receptor is associated with its ubiquitination (Abella et al, 2005). However, using anti-ubiquitin antibodies, we observe no increase in Met ubiquitination in Gab1 over-expressing cells when compared to vector controls, indicating that the enhanced rate of degradation was not as a result of increased Met ubiquitination (Figure 5D), nor does it reflect targeting Met to a triton insoluble compartment (Figures S3B)(Urbe et al, 2003). Moreover, Gab1 does not traffic with the Met receptor on endosomes, but instead remains at the plasma membrane (Figure S3C), demonstrating that Gab1 does not directly recruit Met through the endocytic pathway but instead promotes the formation of HGF dependent dorsal ruffles from which Met internalises.

Disruption of dorsal ruffle formation delays Met receptor degradation and alters HGF induced biological responses.

The localisation of Met to the dorsal ruffle microdomain may be coupled to efficient bulk internalisation of the Met receptor. To test if rapid degradation of Met is coupled to the formation of Gab1 dependent dorsal ruffles, we evaluated Met degradation in cells over-expressing the Gab1ΔCrk mutant that fail to promote dorsal ruffles. Using three stable clones of MDCK cells over-expressing Gab1ΔCrk, we show that the initial rate of Met receptor degradation is significantly delayed when compared to MDCK cells over-expressing Gab1. Under steady state conditions, Met levels decrease by 60% within the first 30 minutes of HGF stimulation in cells expressing wt Gab1, whereas, levels of the Met receptor decrease by only 30% in MDCK cells over-expressing Gab1ΔCrk or in MDCK control cells (Figures 6A and S3D). Similarly, Met is degraded more rapidly in HeLa cells over-expressing Gab1 that form dorsal ruffles, when compared to control HeLa cells (Figures 6B-C), or cells expressing a Gab1ΔPH mutant which fails to induce dorsal ruffles (Figures 6B-C and S2C). Hence, these data demonstrate that recruitment of Met to Gab1-dependent dorsal ruffles facilitates rapid Met receptor degradation.

To establish if enhanced Met degradation is coupled to Gab1 over-expression and/or dorsal ruffle formation, we sought to inhibit dorsal ruffle formation without decreasing Gab1 levels and conversely to promote dorsal ruffle formation by an alternative mechanism utilising the Pak1 kinase. The stilbene drug SITS (4-acetamido-4'-isothiocyabatostilbene-2'2-disulfonic acid), which inhibits the Na^+ independent Cl^-/HCO^- ion exchanger, inhibits dorsal ruffle formation in MDCK cells (Dowrick et al, 1993). Pre-treatment of Gab1 over-expressing MDCK cells with SITS, significantly reduces HGF induced dorsal ruffle formation (8 fold, Figures 6D and S4B)

yet peripheral ruffles and lamellipodia still form under these conditions (Figure 6E). We observe a reduction in scatter of colonies of MDCK cells over-expressing Gab1 treated with SITS (Figure 6F) supporting previous observations that SITS treatment diminished HGF induced cell scatter in MDCK cells (Dowrick et al, 1993). SITS does not interfere with Met phosphorylation in MDCK GFP-Gab1 cells in response to HGF (Figure 6G). Hence, the decreased biological response is not due to decreased Met activation. However, in the presence of SITS, we observe a delay in HGF induced degradation of Met by approximately 2 hours (Figure 6G). This correlates with a delay in the peri-nuclear localisation of Met (Figure S4C). In contrast, SITS treatment had no effect on Met degradation and trafficking in HeLa cells, which do not readily form dorsal ruffles in response to HGF (Figures S4D-E). Since SITS is not a general inhibitor of Met phosphorylation or trafficking, we conclude that the ablation of dorsal ruffles in the presence of SITS, results in an impaired biological response. This is consistent with our previous observations that the Gab1ΔCrk mutant, which is deficient for the induction of dorsal ruffles, also fails to promote cell scatter downstream from Met (Lamorte et al, 2002a).

To establish if enhanced Met degradation was specific only to dorsal ruffles induced following Gab1 over-expression, or was linked to the localisation of Met to dorsal ruffles, we examined Met stability in HeLa cells where dorsal ruffles are induced by Pak1. A Pak1 mutant, which is impaired in its auto-inhibition ability (H83-86L) promotes robust dorsal ruffle formation in response to PDGF (Dharmawardhane et al, 1997). When expressed in HeLa cells, Pak1 H83-86L induces dorsal ruffles in response to HGF (Figure 7A). Immunostaining for endogenous Met revealed that Met was present on the dorsal ruffle membrane (Figure 7A). Importantly, Met was more rapidly degraded in Pak1 H83-86L expressing cells which form dorsal ruffles in response to HGF when compared to vector control cells, which do not form dorsal ruffles (Figure 7B-C). Taken together, these data support that induction of dorsal ruffles per se, in an HGF dependent manner, promotes efficient Met receptor degradation.

Discussion

The physiological significance of dorsal ruffles and their initiation is poorly understood. Our findings demonstrate that the Gab1 scaffold protein is required for dorsal ruffle formation in multiple cell types, including colonies of polarized epithelial cells and downstream from multiple receptor tyrosine kinases (Met, EGFR, PDGFR-β). Gab1 provides a mechanism through which these RTKs couple to signals for dorsal ruffle formation, generating polarised signalling microdomains that contribute to RTK biological responses and RTK down-regulation.

We provide evidence that Gab1 dependent dorsal ruffles function both as a prolonged signalling microenvironment required for epithelial dispersal (Figures 1 C,D, S1 B-D and 6F), but also as a mechanism for bulk internalisation and trafficking of the Met receptor that promotes efficient Met degradation (Figures 5A-C). In the absence of Gab1 over-expression in HeLa cells, Met is internalised by the clathrin dependent pathway, undergoes recycling and is subsequently targeted for degradation (Abella et al, 2005; Hammond et al, 2003). Under these conditions Met is rapidly internalised within 5 minutes post stimulation (Abella et al, 2005; Hammond et al, 2001). Hence, the Met dependent signal at the plasma membrane is transient in nature. In stark contrast, following HGF stimulation of HeLa cells over-expressing Gab1, the activated Met receptor and Gab1 are instead recruited to dorsal ruffles. MDCK cells and HeLa cells transfected with Gab1 continue to form dorsal ruffles up to 20 minutes post-stimulation, providing a prolonged but polarised signalling microenvironment at the plasma membrane, from which Met can be internalised and efficiently degraded (Figures 6B,D, S4A and 8).

The apparent controversy between maintenance of the Met receptor at the plasma membrane in a dorsal ruffle microenvironment versus the shorter half-life of the Met receptor observed in cells that form dorsal ruffles, may reflect that Met is internalised more efficiently from this site and/or that Met fails to recycle. In support of the former, we observe many Met receptor positive vesicles at the base of the dorsal ruffle as it collapses (Figure 1B), as previously reported for the EGF Receptor (Orth et al, 2006). In addition, Met is more rapidly translocated to a peri-nuclear compartment in MDCK cells over-expressing Gab1 that form dorsal ruffles (Figure 5C) consistent with a decrease in recycling and the more rapid degradation observed for the Met receptor (Figures 5A-B). However, it is also possible that Met receptors may first be internalised by a clathrin dependent process and then recycled from an endosomal compartment to the plasma membrane to become incorporated into dorsal ruffles upon HGF stimulation (Figure 8) (Zech & Machesky, 2008).

Multiple mechanisms have been identified for RTK internalisation (Mayor & Pagano, 2007; McNiven, 2006). However, the signals that regulate which mechanism is undertaken by RTKs are still unclear. Met internalisation from the dorsal ruffle microenvironment results in Met localisation onto EEA1 positive endosomes, indicating that dorsal ruffles still deliver receptors to the canonical endocytic pathway (Figure 5C). For the EGFR, ligand concentration can influence the mode of internalisation (Sigismund et al, 2005). Importantly, MDCK cells form dorsal ruffles in response to physiological concentrations of HGF (Figure S1), supporting that entry of Met into the endosomal pathway from a dorsal ruffle microenvironment is a physiologically relevant pathway. We did not observe an increase in Met ubiquitination under conditions of Gab1 over-expression, indicating that enhanced Met ubiquitination was not the signal for the more efficient degradation observed (Figure 5D). In fact, Met ubiquitination was consistently decreased in conditions with Gab1 over-expression, suggesting that Gab1 may compete for binding to Met with Cbl, the E3-ligase for Met, as both are recruited through the

adapter protein Grb2 (Peschard et al, 2001). Cbl has been localised to dorsal ruffles (Scaife et al, 2003), but whether it functions both as an ubiquitin ligase and/or a scaffold protein remains to be tested.

A biological function for dorsal ruffles has remained elusive. Our data support a role for the formation of a dorsal ruffle signalling microenvironment in facilitating the dispersal of colonies of epithelial cells. Expression of the Gab1ΔCrk mutant in MDCK cells blocks dorsal ruffle formation and we have previously shown that this mutant impairs cell dispersal (Figure 6A) (Lamorte et al, 2002a). Inhibition of dorsal ruffle formation with the stilbene drug SITS, inhibits scatter of epithelial colonies in response to HGF, independent of Gab1 over-expression (Figure 6F) (Dowrick et al, 1993), whereas SITS treatment did not block peripheral ruffle or lamellipodia formation (Figure 6E). Interestingly, the matrix metalloproteinease MMP2, has been localised to the tips of dorsal ruffles (Suetsugu et al, 2003), giving rise to the possibility that dorsal ruffles may also promote degradation of the extracellular matrix, to allow for three-dimensional cell migration. This may reflect the inability of the Gab1ΔCrk mutant to promote branching morphogenesis in three-dimensional matrix (Lamorte et al, 2002a).

Gab2, a related protein, regulates Fcγ receptor-mediated phagocytosis in macrophages (Gu et al, 2003) which is required for the internalisation of antigen upon engagement with cell surface receptors. Gab2 localises to nascent phagosomes and by the subsequent recruitment of p85, functions to amplify PIP_3 production required for phagocytosis (Gu et al, 2003). However, we show that for dorsal ruffle formation, the recruitment of p85 to Gab1 is not essential, although pre-treatment of MDCK cells with inhibitors of PI3K blocks the formation of dorsal ruffles (data not shown) consistent with previous studies (Dharmawardhane et al, 1997; Doughman et al, 2003; Orth et al, 2006). Moreover, HGF induced dorsal ruffles appear to be PIP_3 rich as determined by the localisation of a GFP-tagged Akt PH domain, which specifically binds PIP_3 (data not shown). Hence, sources of PI3K activity distinct from Gab1 and possibly recruited directly by the Met receptor (Maroun et al, 1999a) are sufficient for dorsal ruffle formation. In contrast to Gab2 dependent phagosome formation, a Gab1 protein uncoupled from recruitment of the Crk adapter protein fails to promote dorsal ruffles downstream from Met (Figure 4A-C). At the molecular level, the formation of dorsal ruffles is known to be Rac dependent (Krueger et al, 2003; Lanzetti et al, 2004) and at least in part dependent on the activity of WAVE-1, that connects Rac to the actin nucleating complex (Eden et al, 2002; Suetsugu et al, 2003). CrkI/II proteins couple upstream activators to Rac (Feller, 2001) and the Gab1-Crk complex promotes Rac activation in response to HGF (Lamorte et al, 2002a; Lamorte et al, 2002b), hence providing a mechanism for Rac activation in the dorsal ruffle membrane microdomain.

Since the balance of RTK activation and degradation is critical for normal physiology, a full understanding of the molecular events that control these processes is essential. In this framework, Gab1 had always been considered only as a positive regulator of RTK signalling (Birchmeier et al, 2003; Gu & Neel, 2003). In this context, Gab1 and signalling proteins recruited to Gab1 following RTK activation are present on dorsal ruffles providing a prolonged signalling microenvironment. Paradoxically, positive regulation of dorsal ruffles by Gab1 also induces more efficient degradation of the Met receptor and Gab1 may thus play a key role in recruiting RTKs into dorsal ruffles for their subsequent ligand dependent down-regulation. This highlights an unsuspected role for Gab1 in RTK homeostasis.

Materials and Methods

Reagents, Antibodies, Cell culture and Transfections

A detailed list of all plasmids, antibodies, reagents, cell lines and transfection protocols is provided in the Supplemental Materials and Methods.

Growth factor stimulation, Immunoprecipitation and Western blotting

Receptor degradation assays were carried out using HGF (0.46nM or 48ng/ml) in the presence of cycloheximide (100ng/ml). Where indicated, cells were pre-treated with DMSO or SITS (0.5mM) for 20 minutes prior to stimulation. MEF wt and *Gab1*-null were serum starved overnight and stimulated with EGF (5nM or 30ng/ml) or PDGF-bb (11nM or 10ng/ml) for 5 minutes. HeLa and MDCK cells were harvested in TGH lysis buffer, to detect Met receptor ubiquitination in HeLa cells, cells were lysed 24 hours post transfection in RIPA lysis buffer (for details see supplemental Materials and Methods). Cells harvested under boiling lysis conditions were lysed in 200 µl boiling buffer (2% SDS, 1 mM EDTA). Lysates were boiled for 10 min and diluted to 1 ml with a buffer containing 2.5% Triton, 12.5 mM Tris pH 7.5, 187.5 mM NaCl. MEF wt and *Gab1*-null cell lines were lysed in RIPA lysis buffer. Densitometric analysis of western blots were performed using NIH Image J software. Where indicated, data from densitometric analysis was processed using Prism 4.9 to generate a one phase exponential decay best fit curve in order to determine the receptor half-life ($t_{1/2}$) with an error using a 95% confidence interval.

Confocal Immunofluorescence microscopy

MDCK and HeLa cells were seeded at $2x10^4$ on glass cover slips (Bellco Glass Inc. Vineland, NJ) in 24 well plates (Nalgene NUNC, Rochester, NY) and 24 hours later stimulated with 0.46nM HGF or 5nM EGF. Where indicated, cells were pre-treated with DMSO or SITS (0.5mM) for 20 minutes prior to growth factor stimulation. Wild-type and *Gab1*-null MEF were seeded at 1 $x10^4$ on glass cover slips and 24 hours later were serum starved for 16 hours prior to stimulation with 5nM EGF or 11nM PDGFbb. To study Met trafficking, MDCK and HeLa cells were serum starved for 2 hours in the presence of cycloheximide prior to HGF stimulation. Cover slips were washed twice with PBS, fixed with 2% paraformaldehyde (PFA, Fisher Scientific). Staining procedures have previsously been described in (Abella et al, 2005). Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope (Carl Zeiss, Canada Ltd, Toronto, ON) with 100X or 40X objective. Image analysis was carried out using the LSM 5 image browser (Empix Imaging, Mississauga, ON). Confocal live cell imaging was performed either using a Zeiss 510 Meta laser scanning confocal microscope or with Spinning disk confocal microscope from Quorum Technologies. Image analysis from data acquired using the LSM5 microscope was performed on LSM 5 image browser. Data from the spinning disk microscopy was analysed using Volocity 4.1 software.

Dorsal Ruffle Assays

Cells plated on coverslips were stimulated with growth factor for 5 minutes, fixed in 2% PFA and stained with Phalloidin Alexa-Fluor 488 or 545. Using a confocal microscope with a 40x objective, the number of cells which formed dorsal ruffles were counted and represented as percent of the total number of cells counted. At least 10 fields of view were counted for each

experiment. For Gab1 siRNA experiments, the number of cells forming dorsal ruffles was scored from a minimum of 12 different fields of view (>650 cells) were scored and represented in a histogram as fold change in dorsal ruffle formation compared to mock transfected cells.

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Figure Legends.

Figure 1. The Met receptor and the Gab1 scaffolding protein are recruited to dorsal ruffles. (A) HGF induces peripheral and dorsal ruffle formation in MDCK cells. MDCK cells were stimulated or not with HGF for 5 minutes, fixed and stained with Phalloidin-Alexa Fluor 488 and dapi. Solid arrows delineate dorsal ruffles and dashed arrows peripheral ruffles. (B) The Met receptor is recruited to dorsal ruffles in MDCK cells. MDCK Met cells were stimulated or not with HGF for 5 minutes, fixed and stained for Met (red) and Phalloidin-Alexa Fluor 488. Bottom panel is a yz section plane through the dorsal ruffle. MDCK Met/HA-Gab1 cells were stimulated or not with HGF for 5 minutes fixed and stained for (C) phospho-Met (pY1234/35) (red) or (D) for phospho-Erk1/2 (red) and HA (green). Confocal images were taken with a 100x objective. Bar represents 10 μm.

Figure 2. Gab1 regulates dorsal ruffle formation downstream of the Met receptor. (A) MDCK cells, mock, or transfected with scrambled siRNA or three different siRNA duplexes targeting Gab1 were assayed for their ability to make dorsal ruffles. Gab1 protein knock down is shown in inset by western blot analysis 96hrs post transfection. The graph is a representative of three independent experiments. Significant decreases in dorsal ruffle formation are denoted by p-values, where no significant differences were observed in the scramble condition as compared to mock. (B) MDCK and MDCK GFP-Gab1 were stimulated with HGF for 5 minutes, fixed and stained with Phalloidin Alexa-Fluor 546. (C) Protein extracts from MDCK and MDCK GFP-Gab1 cells were separated by SDS PAGE and immunoblotted for Gab1, GFP and actin to determine the extent of Gab1 expression. (D) Cells forming dorsal ruffles were scored from 10 individual fields in MDCK ( ) and MDCK GFP-Gab1 ( ) cells at 0, 5 and 10 minutes post HGF stimulation. Values represent the mean + S.E.M. of three separate experiments. (E) HeLa cells transiently transfected with HA-Gab1 were stimulated or not with HGF, fixed and stained for HA (red) to detect transfected cells and Phalloidin-Alexa Fluor 488 to detect the presence of dorsal ruffles (* denotes untransfected cell).

Figure 3. Gab1 is required for dorsal ruffle formation downstream from the EGF and PDGF receptors.
(A) The ability of wild-type MEF (6B) and two clones of MEF cells from *Gab1*-null animals (7C and 2B) to form dorsal ruffles in response to EGF and PDGFbb was examined. Cells were serum starved, and stimulated for 5 minutes with indicated ligand. Dorsal ruffle formation is scored based on co-staining with cortactin (red) and Phalloidin-Alexa Fluor 488. (B) The percentage of cells capable of forming dorsal ruffles was scored from 10 individual fields in the unstimulated condition ( ) PDGF ( ) or EGF ( ). Values represent the mean + S.E.M. of three independent experiments. (C) Re-expression of Gab1 in *Gab1*-null MEF rescues dorsal ruffles formation. Both clones of *Gab1*-null MEF were transfected with GFP-Gab1 for 24 hours, starved and stimulated for 5 minutes with indicated ligand. The actin cytoskeleton is visualized with Phalloidin-Alexa Fluor 546 and representative pictures are shown of MEF 7C. All scale bars represent 10μm.

Figure 4. A Gab1-Crk complex is required for dorsal ruffle formation downstream of Met.

(A) HeLa cells transfected with HA-tagged Gab1 or Gab1 mutants (Gab1ΔShp2, Gab1Δp85, or Gab1ΔCrk) were stimulated with HGF for 5 minutes, fixed and stained for HA (red) and Phalloidin-Alexa Fluor 488 to detect dorsal ruffles. Representative images from four separate experiments are shown. (B) MDCK cells, or MDCK cell lines that stably express HA-tagged Gab1 wt, or Gab1 mutants were left unstimulated or stimulated for 5 minutes with HGF, fixed stained with Phalloidin-Alexa Fluor 488. Representative confocal images of the most apical z-section are shown. (C) The percentage of cells which form dorsal ruffles in (B) was scored where cells were left unstimulated ( ) or treated for 5 minutes with HGF ( ) and the average response is plotted + S.E.M. (D) Two MDCK cell lines over-expressing CrkII (B1 and C9) were quantified for their ability to form dorsal ruffles without stimulation ( ) or following 5 minutes with HGF ( ) and the average response plotted + S.E.M. Inset represents protein levels of CrkII in the cell lines used. All scale bars represent 10μm.

Figure 5. Dorsal ruffle formation enhances Met receptor degradation.
(A) MDCK and MDCK HA-Gab1 expressing cells were stimulated with HGF for the indicated times. Proteins from cell extracts were separated by SDS PAGE and immunoblotted for Met, HA-Gab1 and actin. (B) Densitometric analysis of Met degradation as percentage of initial receptor remaining after HGF stimulation +/- S.E.M. as in (A) from three independent experiments was used to generate a best fit one phase decay curve to determine the half life ($t_{1/2}$) of the receptor. (C) MDCK Met and MDCK Met/HA-Gab1 cells were serum starved in the presence of cycloheximide, stimulated with HGF for the indicated time points, fixed and stained for Met (red), EEA1 (green) and dapi (blue). Confocal images were acquired with 100x objective and the outline of the cells in the stimulated conditions, were drawn using the DIC image as a guide. (D) HeLa cells transiently transfected with vector or increasing amounts of HA-Gab1, were stimulated or not with HGF for 5 minutes and lysed in RIPA buffer. Met protein was immunoprecipitated, separated by SDS PAGE and immunoblotted for ubiquitin, stripped and reprobed for Met (147). Total cell lysates were immunoblotted for HA-Gab1.

Figure 6. Dorsal ruffles are required for Met down-regulation and biological activity.
(A) MDCK, MDCK HA-Gab1 cells, and three cell lines expressing HA-Gab1ΔCrk were stimulated with HGF for the indicated times. Densitometric analysis from three independent experiments is presented as percentage of initial receptor remaining after HGF stimulation +/- S.E.M. (B) HeLa cells were transiently transfected with vector, HA-Gab1 or HA-Gab1ΔPH, and 24 hours later stimulated with HGF for indicated times. Proteins from cell lysates were separated by SDS PAGE and immunoblotted for Met, HA-Gab1 and actin. (C) Densitometric analysis of Met degradation presented as percentage of initial receptor remaining after HGF stimulation, +/- S.E.M. as in (B) from three independent experiments. (D) MDCK GFP-Gab1 cells were pre-treated with DMSO or 0.5mM SITS and stimulated with HGF. The number of cells forming dorsal ruffles was scored from 10 fields of view at 0, 5, 10 and 20 minutes post HGF stimulation, represented as the percentage of cells forming dorsal ruffles over time with DMSO ( ) or SITS ( ) treatment. (E) MDCK GFP Gab1 cells were pretreated with 0.5mM SITS then stimulated with HGF and imaged under live conditions. Confocal images of different cells taken with a 100X objective are shown at the indicated time points. The outline of the cells was drawn using the DIC images as a guide. Scale bar represents 10μm. (F) MDCK GFP-Gab1 cells were pre-treated with DMSO or 0.5mM SITS and stimulated or not with HGF. Phase contrast images were taken 24 hours post stimulation. Scale bar represents 100μm. (G) MDCK GFP-Gab1 cells were

pre-treated with DMSO or 0.5mM SITS and stimulated with HGF for the indicated times. Cell lysates were immunoblotted for endogenous Met receptor, pY1234/35 Met, GFP and actin.

Figure 7. Pak1 mediated dorsal ruffles enhance Met receptor degradation.
(A) HeLa cells transiently transfected with myc-Pak1 H83-86L were stimulated or not with HGF for 15 minutes, fixed and stained for endogenous Met receptor (red) and myc (green). (B) HeLa cells transiently transfected with vector or myc-Pak1 H83-86L, were stimulated with HGF for indicated times. Proteins from cell lysates were separated by SDS PAGE and immunoblotted for endogenous Met receptor, myc-Pak1 and actin. (C) Densitometric analysis of Met degradation presented as a percentage of initial receptor remaining after HGF stimulation as in (B) +/- S.E.M. from three independent experiments.

Figure 8. Gab1 promotes RTK induced dorsal ruffles which mediate receptor signalling and more efficient down-regulation.
Gab1 switches Met internalisation from clathrin mediated endocytosis to dorsal ruffles upon HGF stimulation. Here, activated Met receptors localise with Gab1 and Gab1 recruited signalling molecules (p85,Crk and Shp2). This microenvironment induces local activation of Erk1/2. Upon collapse of dorsal ruffles, Met receptors are internalised and traffic to a peri-nuclear compartment where Met receptor degradation is more efficient downstream from dorsal ruffles. Importantly, the Gab1 scaffold remains at the plasma membrane. It is not clear whether Met receptors internalised through clathrin coated pits can be recycled to membranes where dorsal ruffles form. EEA1 (Early endosomal antigen 1).

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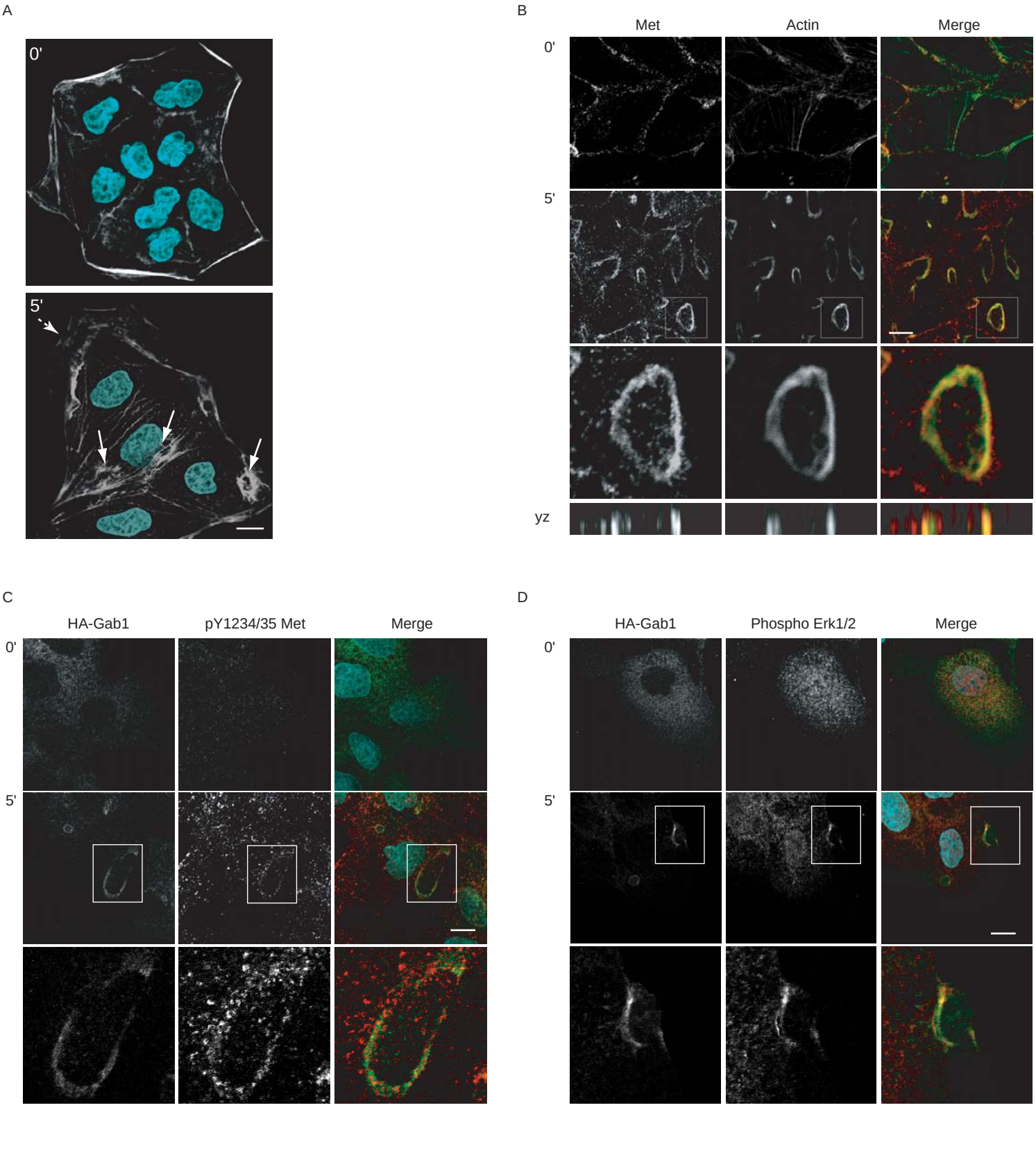

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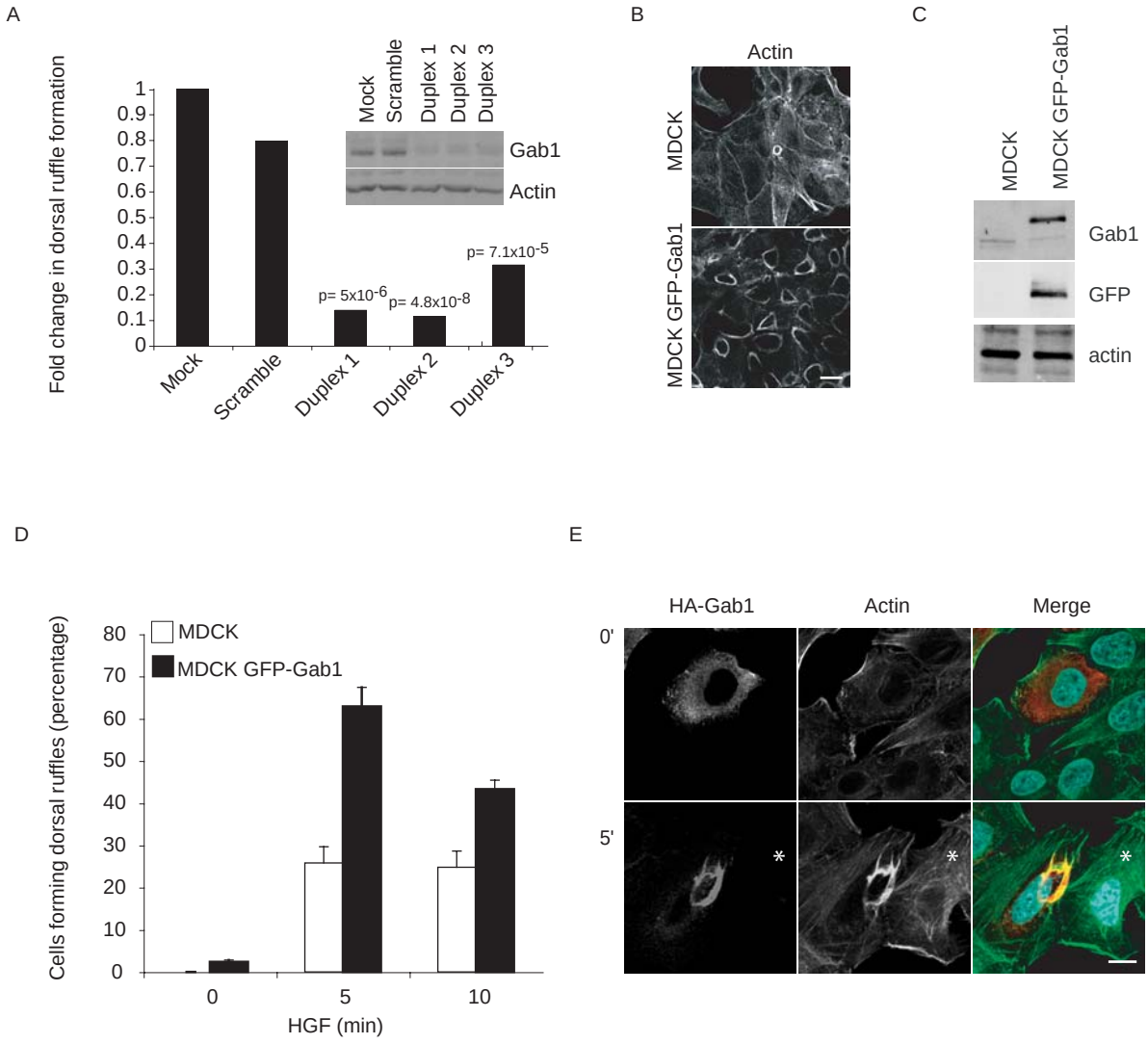

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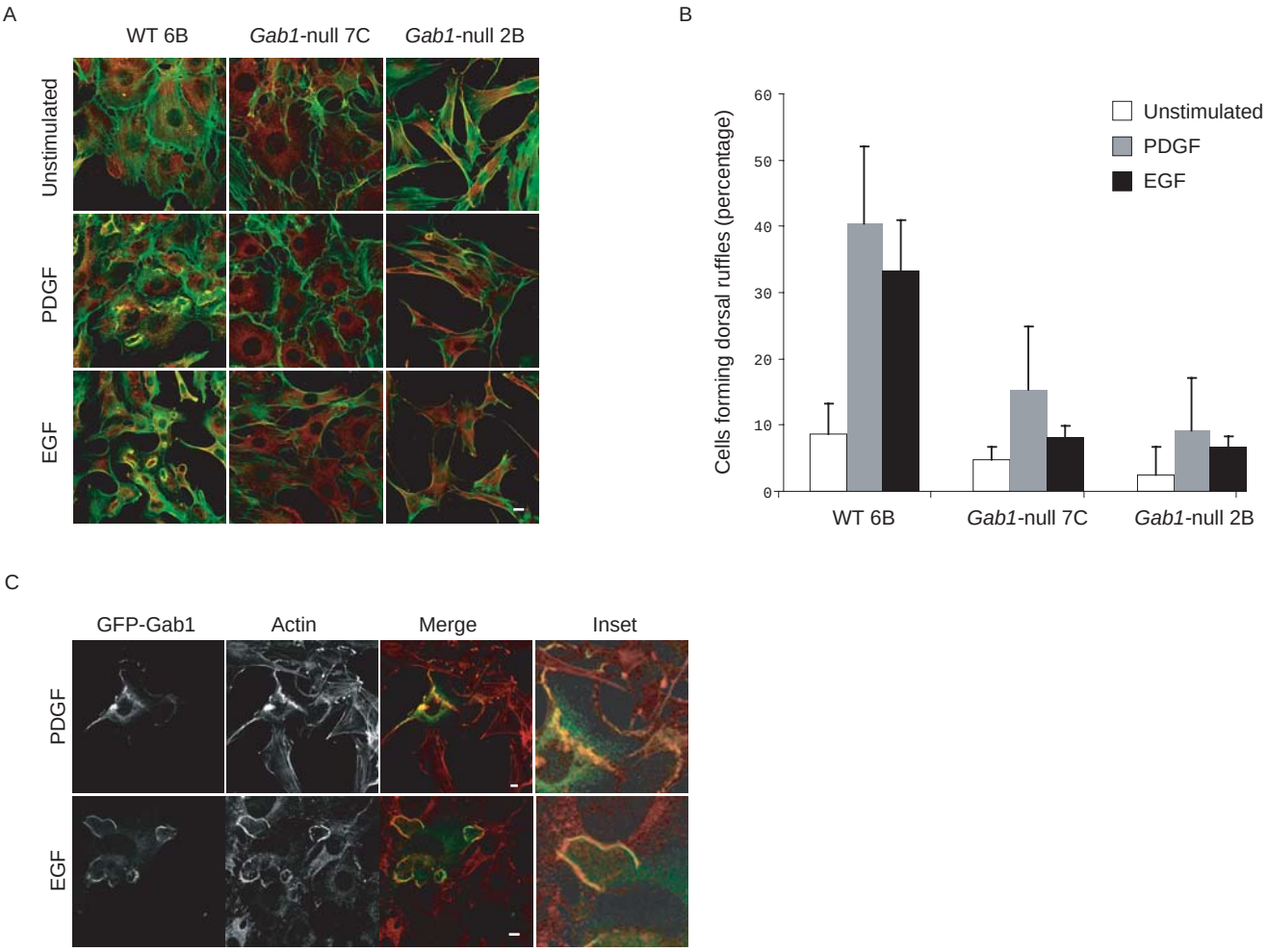

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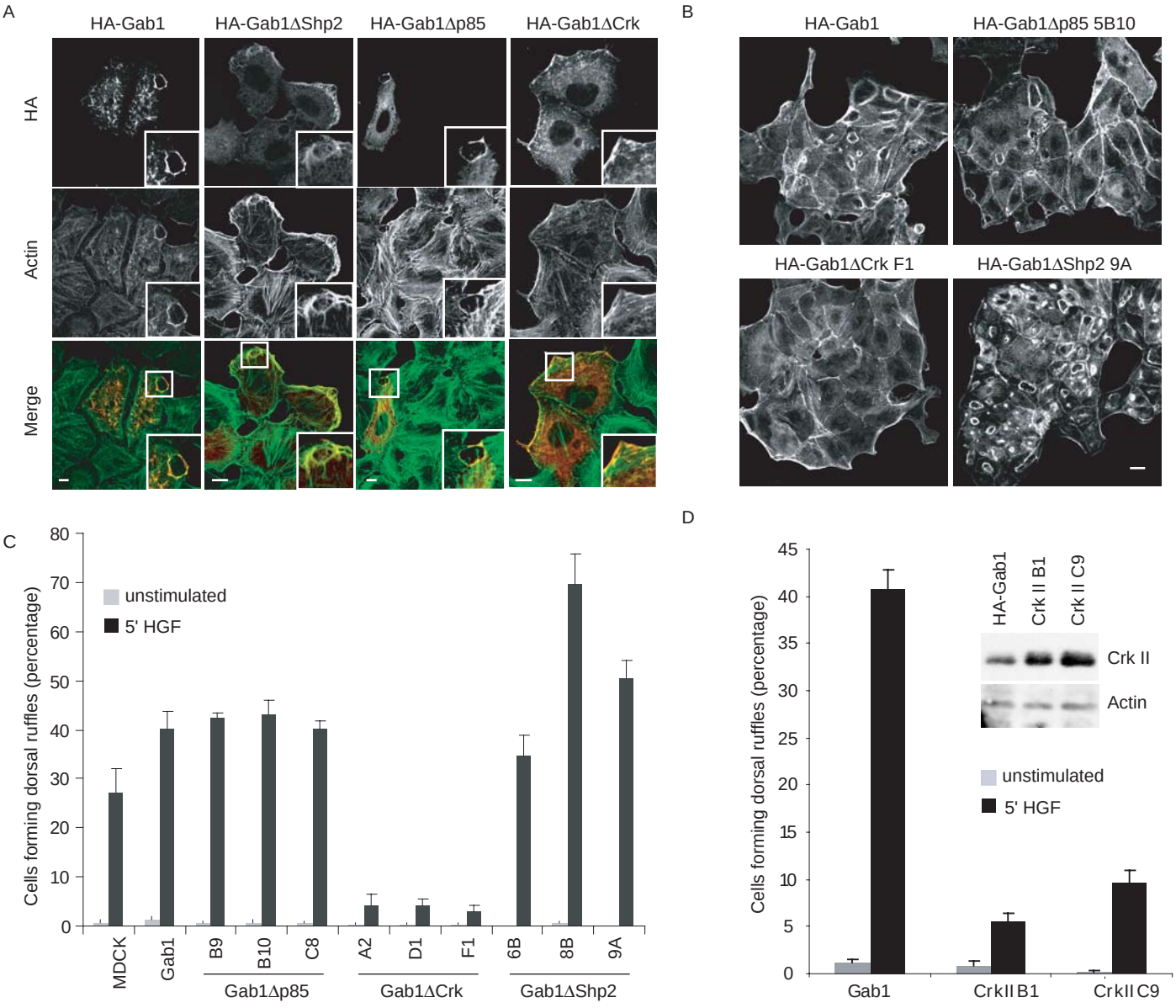

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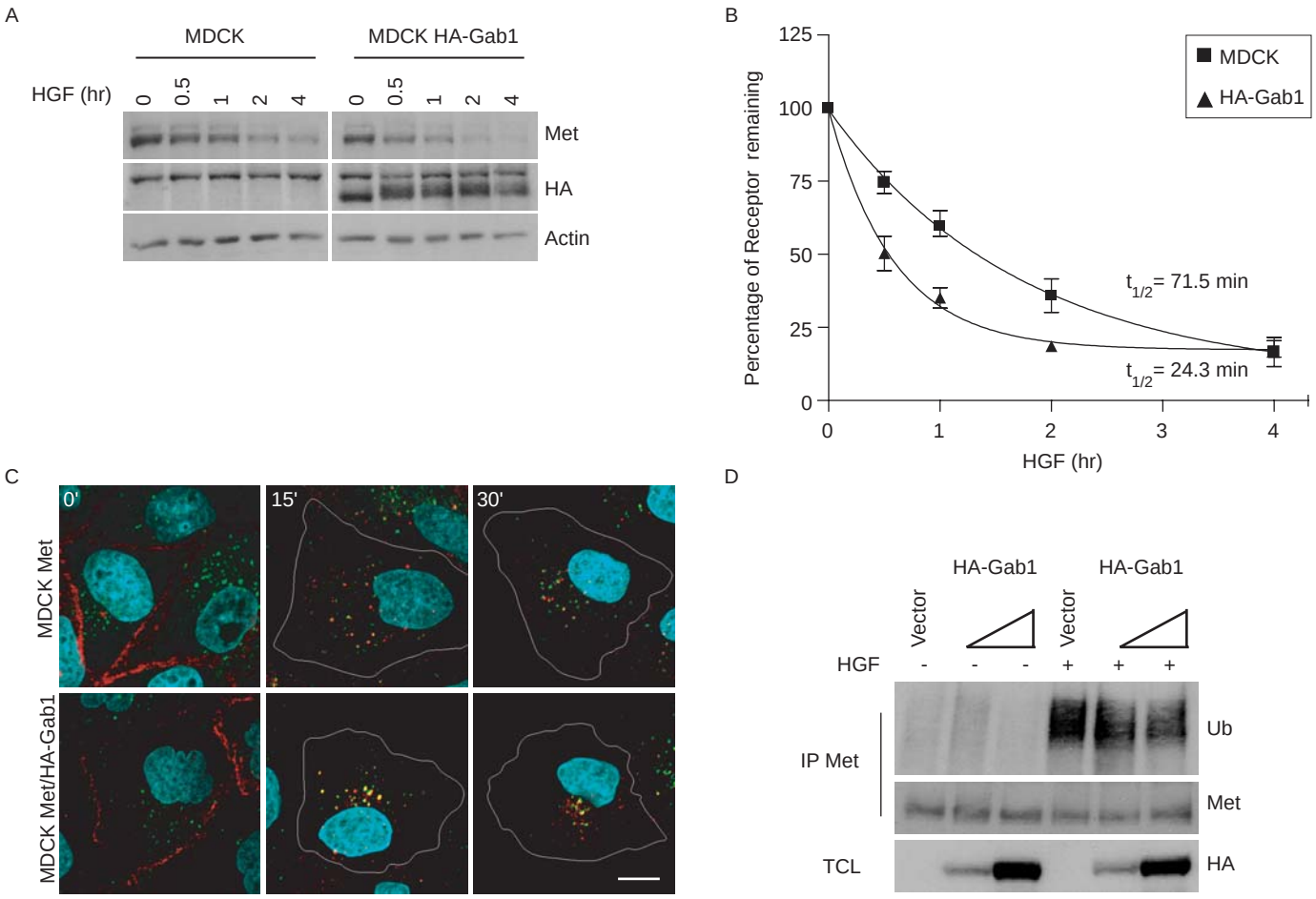

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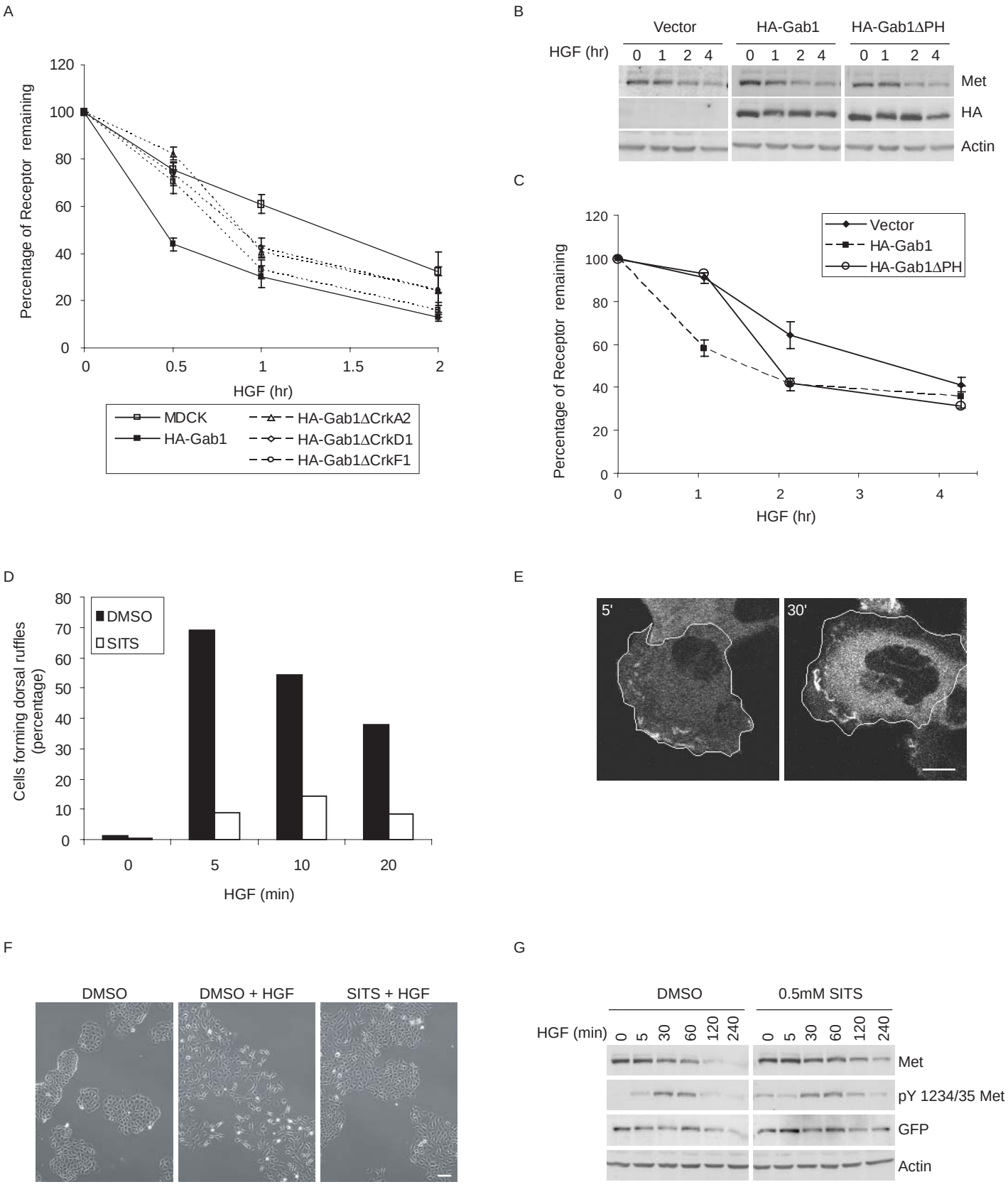

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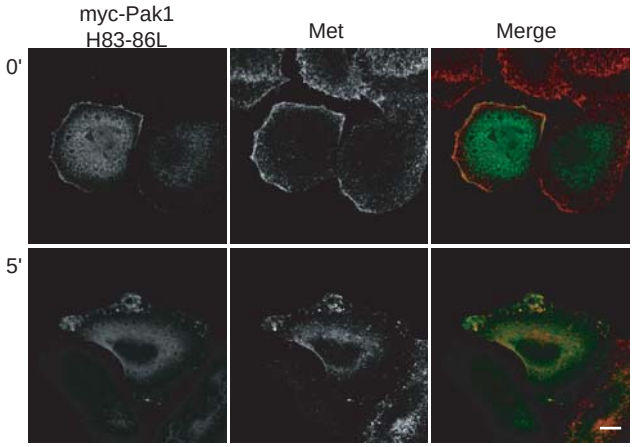

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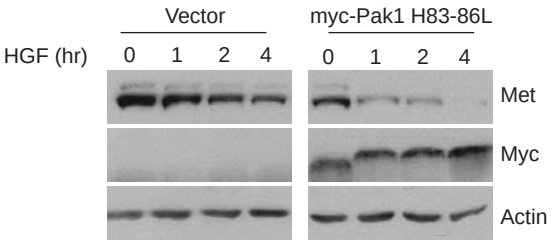

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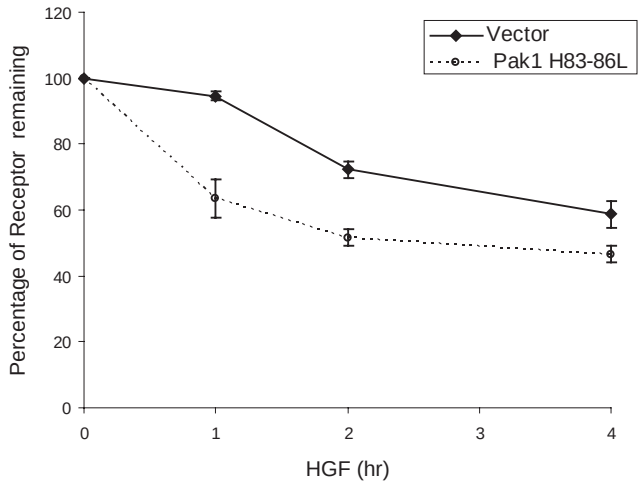

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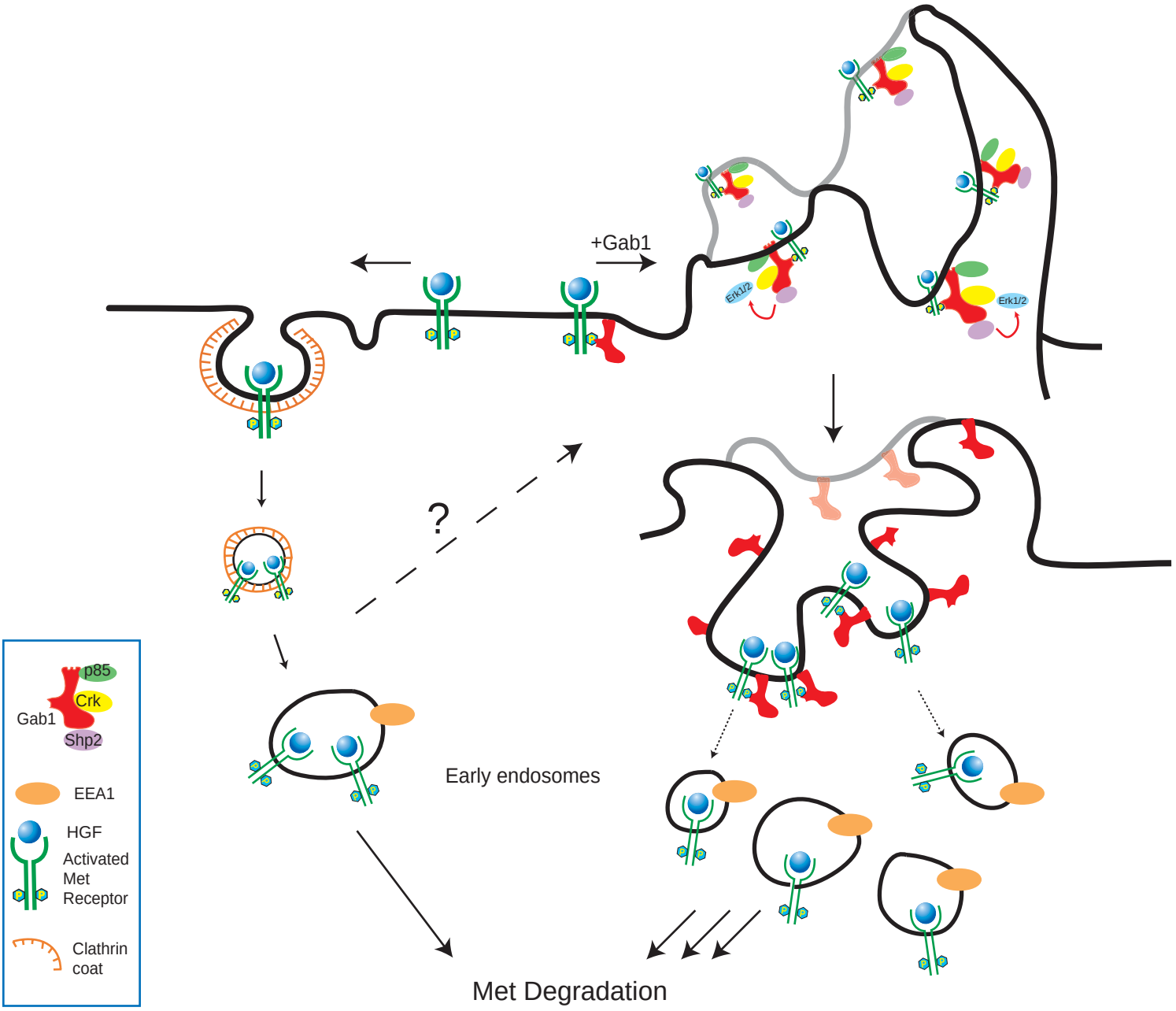

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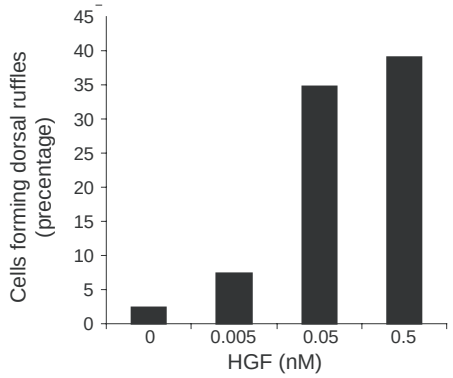

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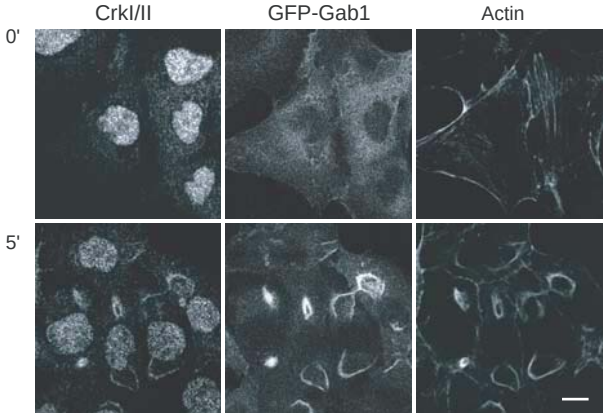

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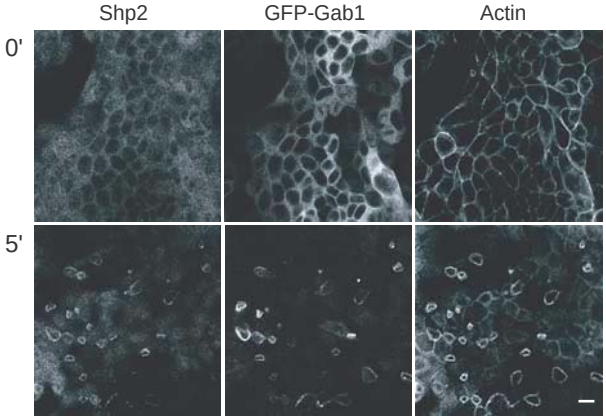

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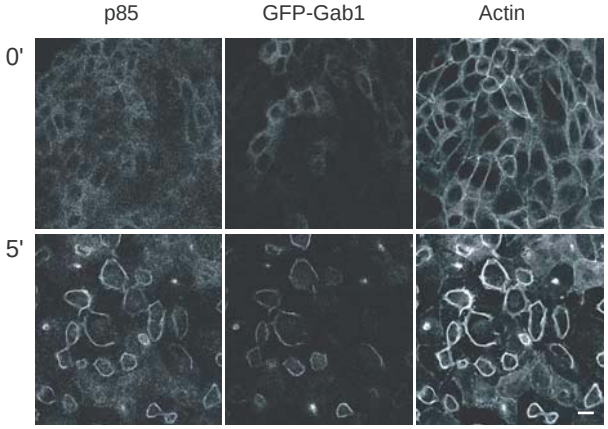

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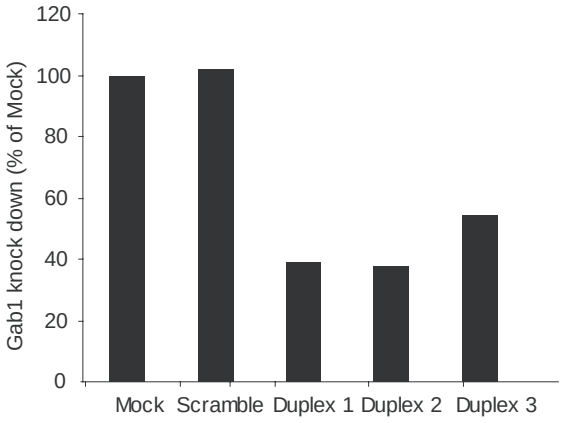

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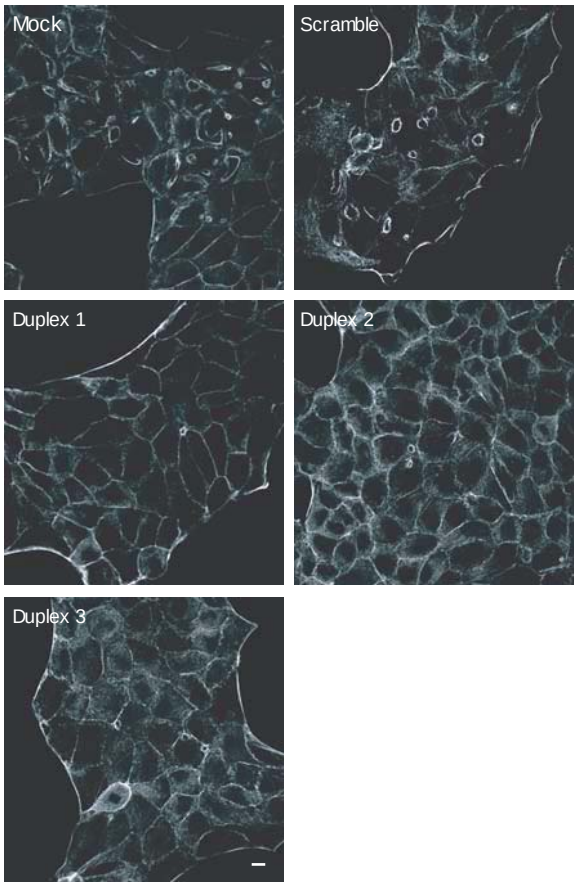

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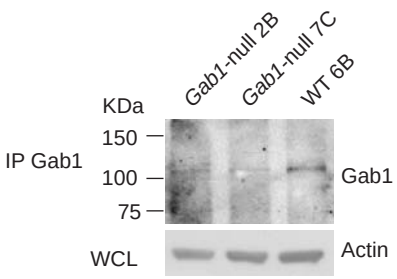

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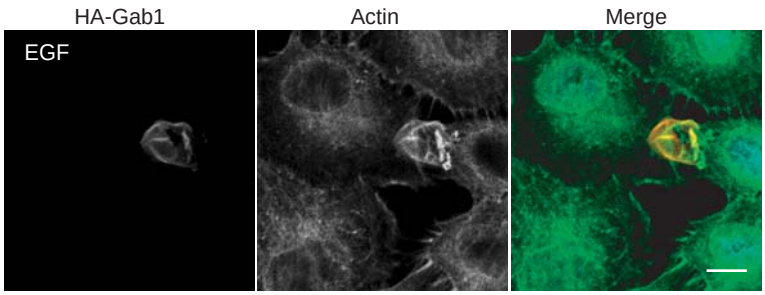

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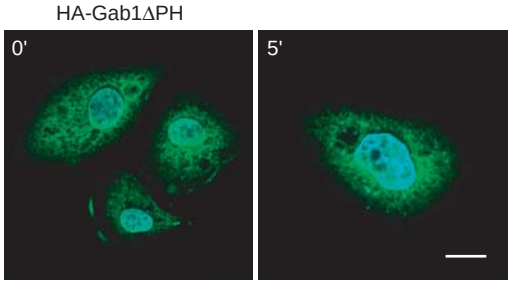

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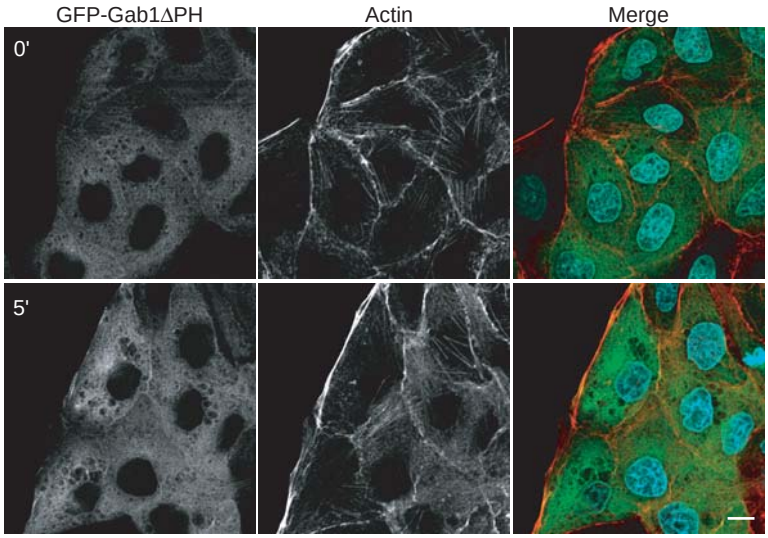

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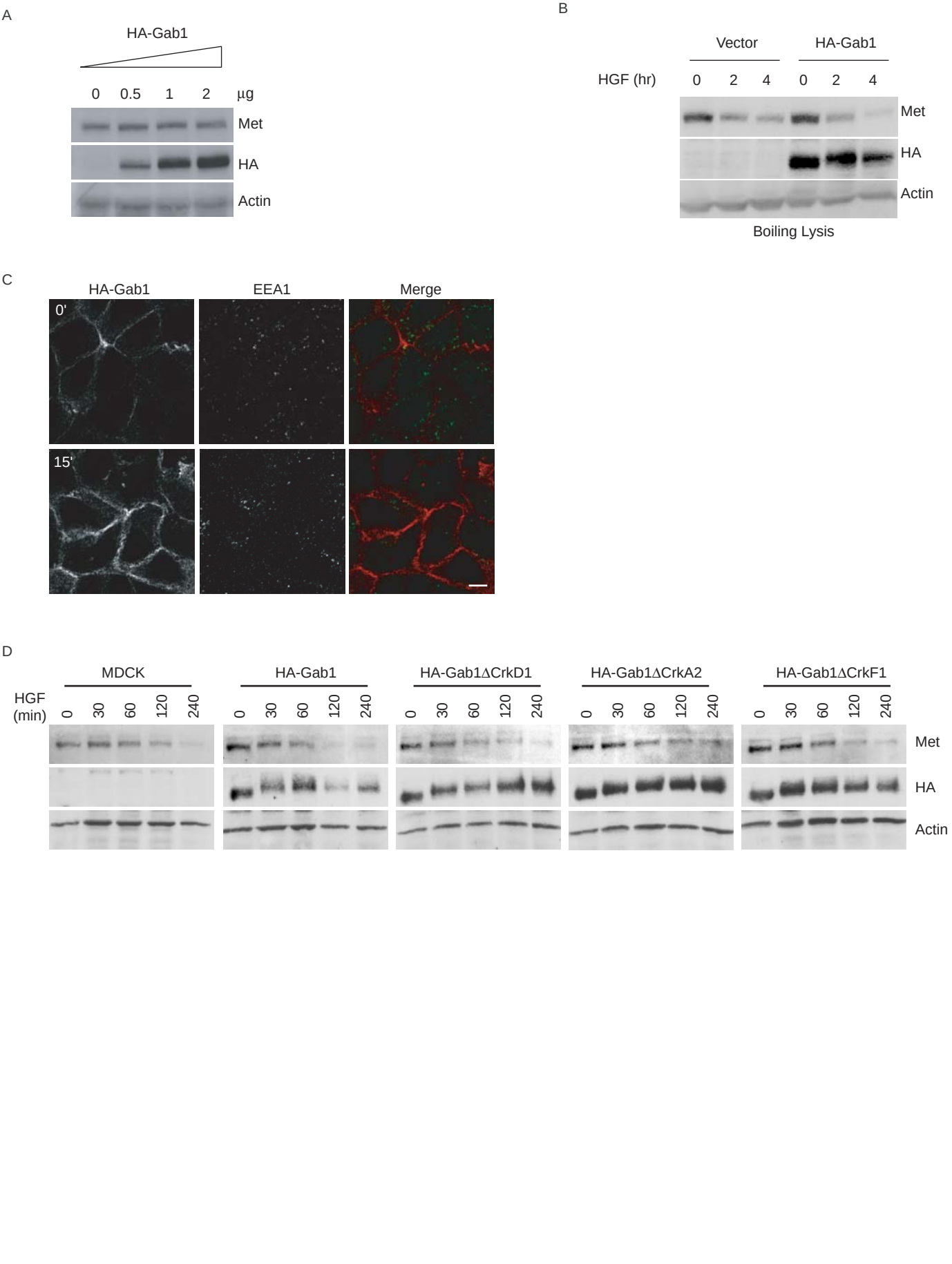

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